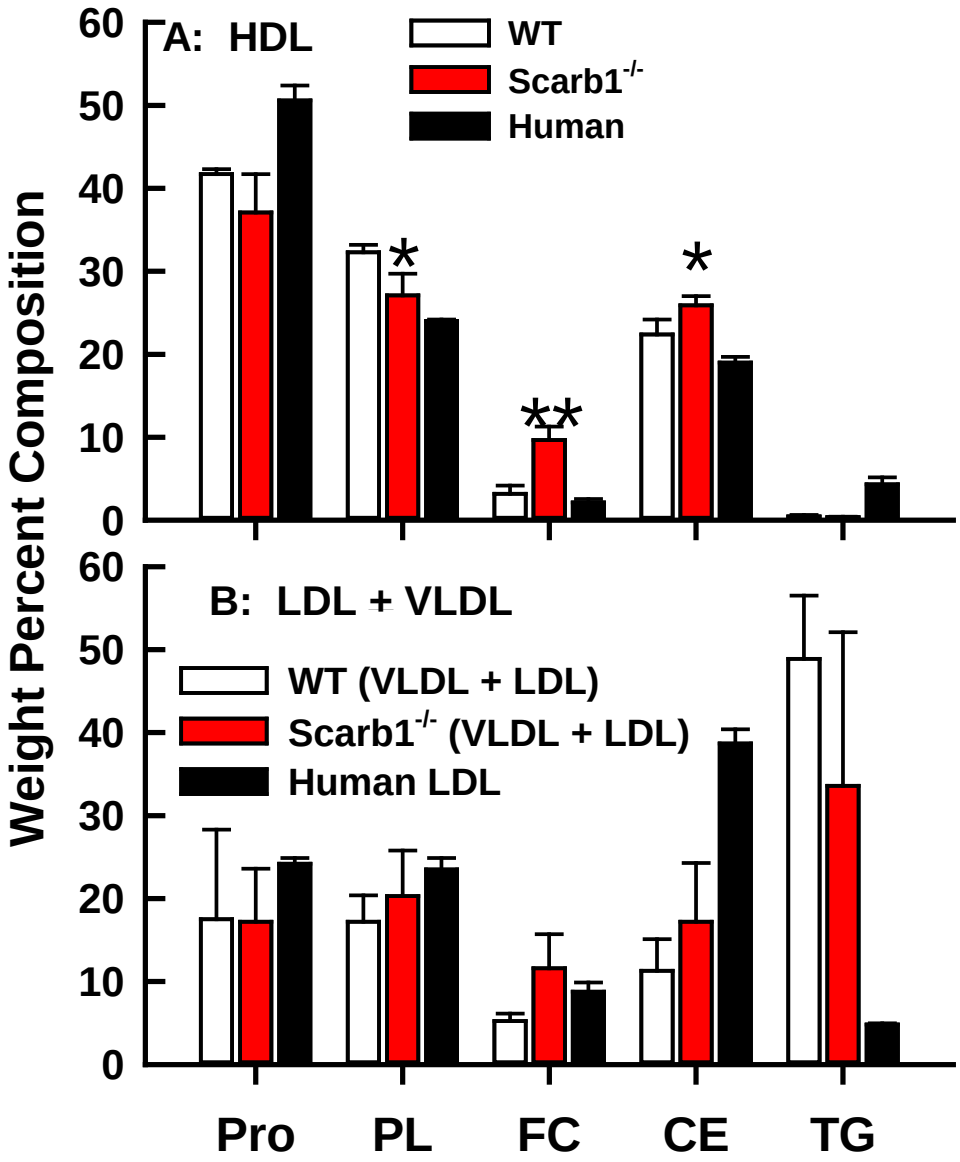
Supplemental Materials

Jing Liu,[1, 2] Baiba K. Gillard,[2, 3*] Dedipya Yelamanchili,[2]

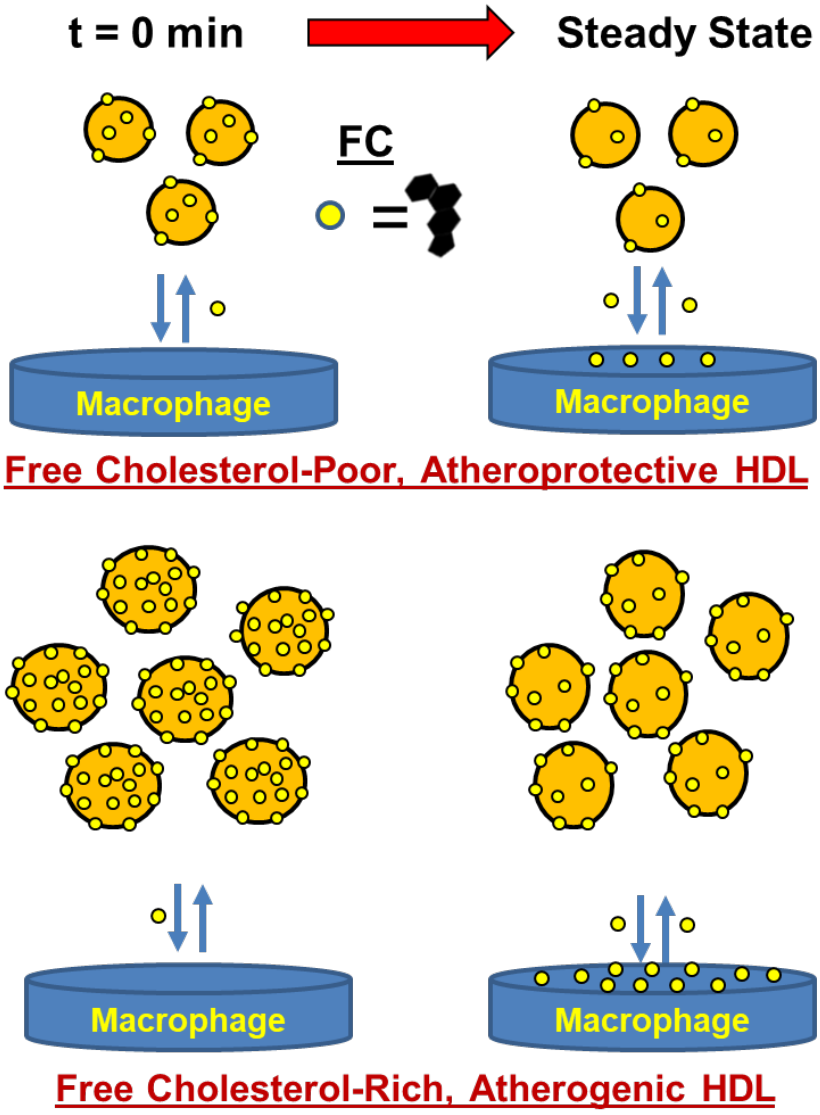
Antonio M. Gotto Jr.,[2, 3] Corina Rosales,[2, 3] and Henry J. Pownall [2, 3*]

High Free Cholesterol Bioavailability Drives the Tissue Pathologies in Scarb1$^{-/-}$ Mice

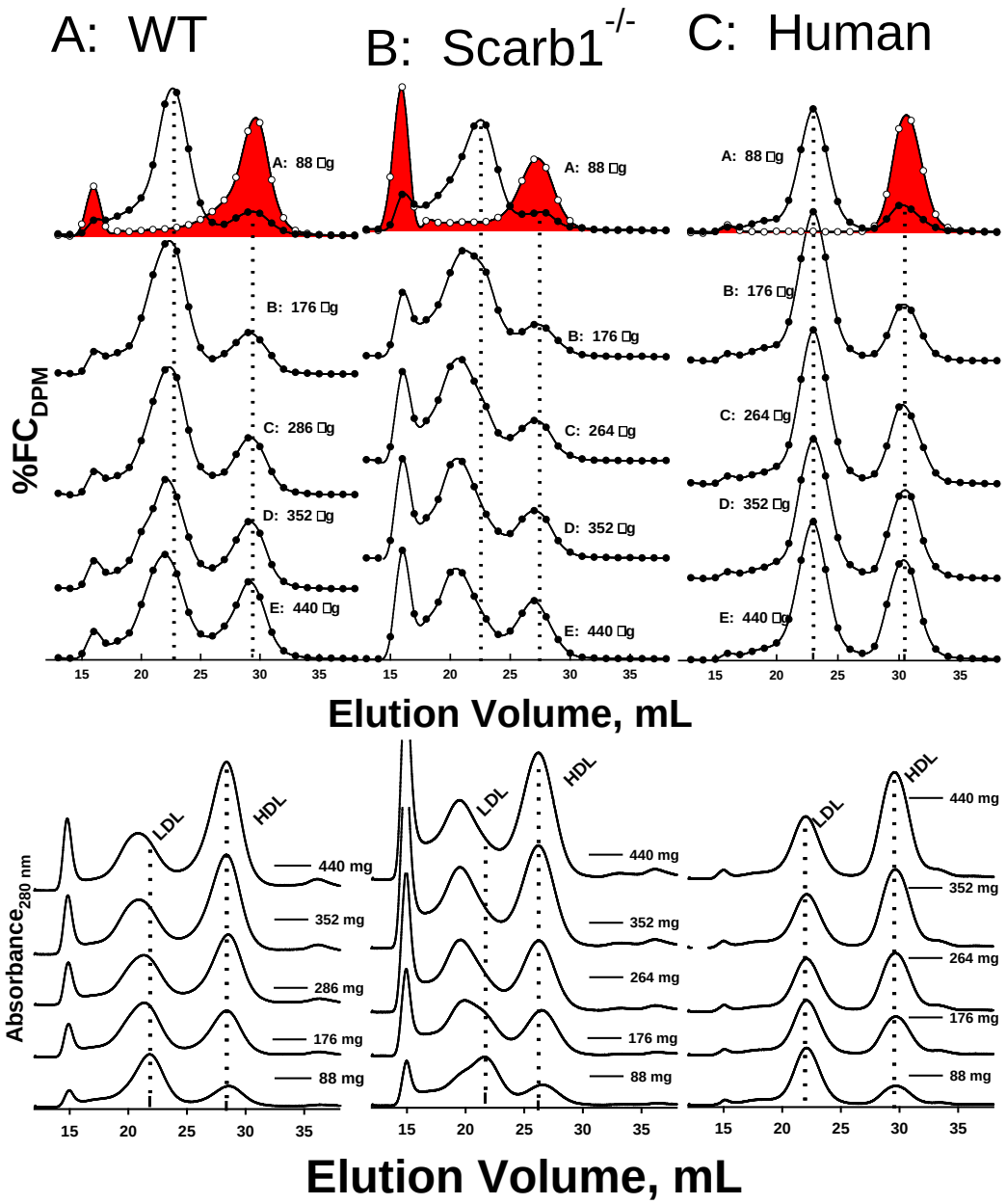
[1]Department of Cardiovascular Medicine, Xiangya Hospital, Central South University, Changsha 410008, China, [2]Center for Bioenergetics, Houston Methodist Research Institute, 6670 Bertner Avenue, Houston TX 77030, USA; [3]Department of Medicine, Weill Cornell Medicine, 1300 York Ave, New York, NY, 10065, USA.



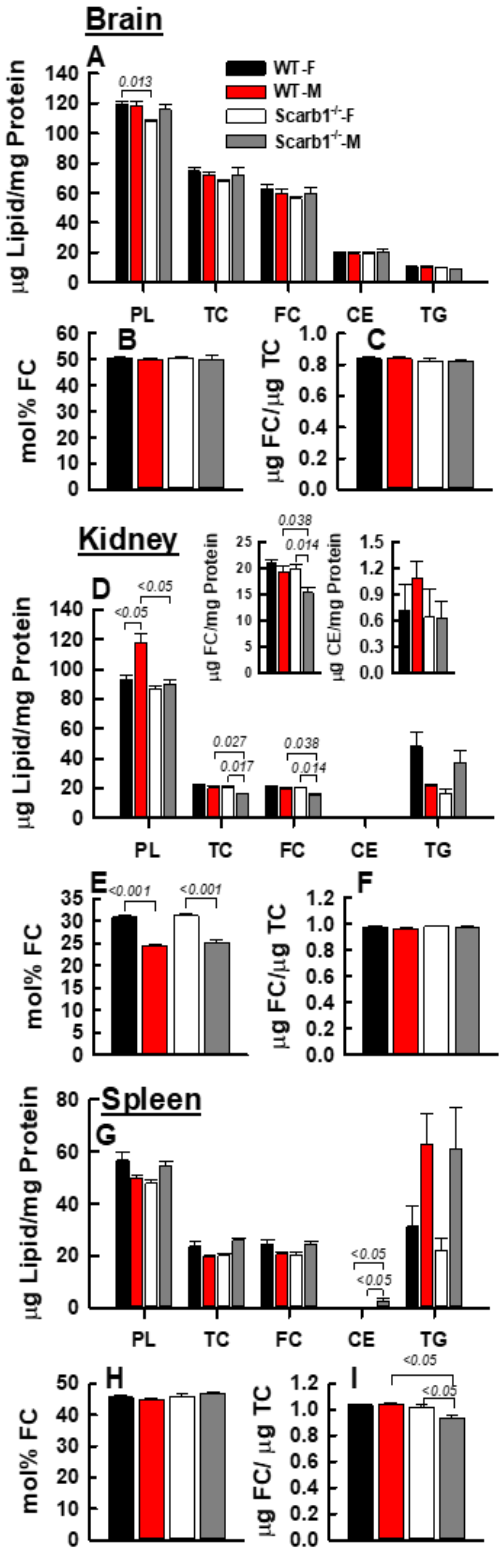
Supplementary Figure I: Lipoprotein Compositions. A) Human and Scarb1$^{-/-}$ mouse HDL. B) Human LDL and Scarb1$^{-/-}$ and WT mouse (VLDL + LDL). *P < 0.05 for Scarb1$^{-/-}$ vs. WT lipoprotein. **P < 0.001 Scarb1$^{-/-}$ vs. WT. This bar graph was created from the data in Supplementary Table 1.



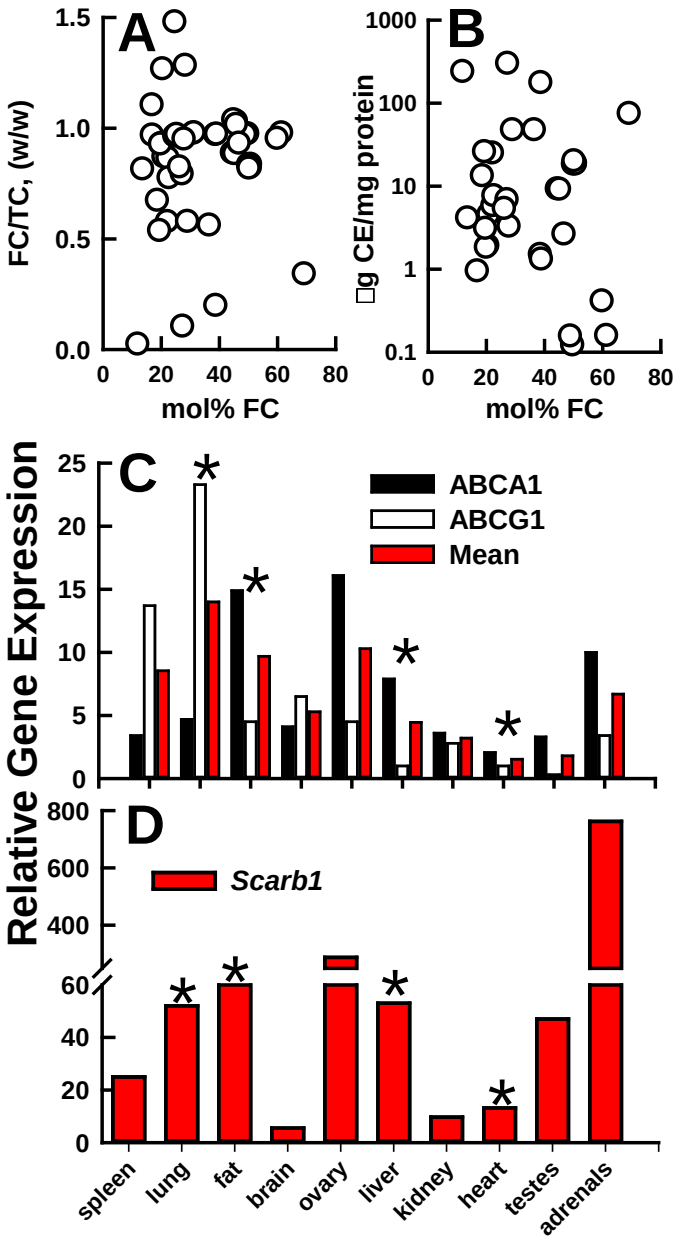
Supplementary Figure II: Hypothetical Mechanism by which HDL-FC Bioavailability Drives FC Transfer into other Lipid Surfaces. Kinetic model compares FC transfer from FC-poor and -rich HDL. Relative numbers of FC on the surfaces for FC-poor and rich HDL are proportional to the reported values for WT and Scarb1$^{-/-}$ mouse HDL. FC distribution is shown at t = 0 and at steady state as labeled.



Supplementary Figure III. Extent of [^{3}H]FC transfer from HDL of WT mice (A), Scarb1$^{-/-}$ mice (B), and humans (C) (88 to 440 µg protein/220 µL) to human LDL (176 µg protein/220 µL) at equilibrium as determined by SEC. Top panels: [^{3}H] FC dpm, bottom panels A_{280nm}. Red chromatograms in the topmost panels (A: 88 ug HDL) are the SEC profiles for the starting radiolabeled HDL.



Supplementary Figure IV: Lipid Compositions of Brain, Kidney, and Spleen of Male (M) and Female (F) WT and Scarb1$^{-/-}$ Mice. Tissue sites are as labeled. Bars are mean ± SEM. Numbers of mice per group for all three tissues are: WT-F (n = 12), WT-M (n = 10), Scarb1$^{-/-}$-F (n = 11) and Scarb1$^{-/-}$-M (n = 5). Statistics as in Methods and **Figure 1** legend. P values for significant differences ($p<0.05$) for comparison of WT vs. Scarb1$^{-/-}$ mice of the same sex, and M vs. F of the same genotype are shown.



Supplementary Figure V. Correlation of tissue FC/TC ratio (A) and CE/protein ratio (B) vs. mol% FC. Data from **Supplementary Table III**. C. Tissue expression of cholesterol transporters ABCA1 and ABCG1. D. Tissue expression of SCARB1. *Denotes tissues that are FC-enriched among Scarb1$^{-/-}$ vs. WT mice. The data show that there is not pattern of all-high or all-low expression of ABCA1, ABCG1, and Scarb1 that associates with FC-enrichment. Gene expression data are from https://www.ncbi.nlm.nih.gov/gene.

Supplementary Table I: Lipoprotein Compositions (weight %)[a]

	n	Pro	PL	FC	CE	TG	Mol% FC
HDL							
WT Mice	4	41.7 ± 0.6	32.3 ± 0.9	3.18 ± 1.01	22.4 ± 1.8	0.43 ± 0.18	16.0 ± 4.3
Scarb1$^{-/-}$ Mice	4	37.1 ± 4.6	27.1 ± 2.6	9.67 ± 1.61	25.9 ± 1.1	0.36 ± 0.05	41.1 ± 2.4
Human	2	50.6 ± 1.8	24.0 ± 0.2	2.15 ± 0.41	19.0 ± 0.7	4.34 ± 0.82	15.0 ± 2.5
t-test p[b]		*0.087*	*0.010*	*0.000*	*0.017*	*0.467*	*0.000*
VLDL+LDL							
WT Mice	3	17.5 ± 10.8	17.2 ± 3.2	5.25 ± 0.89	11.3 ± 3.8	48.9 ± 7.6	37.6 ± 1.4
Scarb1$^{-/-}$ Mice	3	17.2 ± 6.4	20.3 ± 5.5	11.60 ± 4.1	17.2 ± 7.1	33.6 ± 18.5	52.5 ± 2.0
Human LDL	2	24.2 ± 0.7	23.5 ± 1.4	8.79 ± 1.09	38.7 ± 1.7	4.83 ± 0.13	42.3 ± 1.6
t-test p[b]		*0.973*	*0.438*	*0.059*	*0.269*	*0.257*	*0.000*

[a]Mouse HDL and VLDL + LDL lipoproteins were purified from n pools of plasma from 5 - 10 mice each. Human HDL and LDL were from individual donors. Values are mean ± SD. Pro = protein, PL = phospholipid, FC = free cholesterol, CE = cholesteryl ester, TG = triglyceride. [b] Student t-test p value, WT vs Scarb1$^{-/-}$ mice.

Supplementary Table II: Kinetic Constants for FC Flux between HDL and J774 Macrophages[a]

FC Influx					
HDL	Time Course			Dose Response	
	k_i (initial influx) *nmol FC influx/mg cell-protein/min*	k, *min^{-1}*	$Influx_{max}$ *nmol FC/mg cell-protein*	$Influx_{max}$ *nmol FC/mg cell-protein*	$Influx_{50\%}$ *µg HDL-protein/mL*
$Scarb1^{-/-}$	0.098	0.020 ± 0.001	4.9 ± 0.11	26.7 ± 0.7	113 ± 5
WT	0.024	0.019 ± 0.001	1.2 ± 0.02	8.7 ± 0.6	150 ± 14
Human	0.033	0.012 ± 0.002	2.7 ± 0.17	18.3 ± 4.8	136 ± 52
FC Efflux					
HDL	Time Course			Dose Response	
	k_e (initial efflux), *nmol FC efflux/mg cell-protein/min*	k, *min^{-1}*	$Efflux_{max}$ *nmol FC/mg cell-protein*	$Efflux_{max}$ *nmol FC/mg cell-protein*	$Influx_{50\%}$ *µg HDL-protein /mL*
$Scarb1^{-/-}$	0.139	0.018 ± 0.004	7.6 ± 0.9	32.3 ± 2.0	72.1 ± 7.9
WT	0.154	0.019 ± 0.006	8.3 ± 1.1	37.1 ± 2.7	81.2 ± 10.4
Human	0.124	0.016 ± 0.005	7.6 ± 1.1	34.4 ± 3.0	81.8 ± 12.2

[a] Rate constants and maximum influx and effux values were calculated by exponential and hyperbolic fits to the time course and dose response data of **Figure 2 G - J**. Initial rate contants k_i and k_e are calculated from the initial slope of the time course, while k values and influx and efflux maximum and 50% values are from the fit of the data over the entire time course or dose response. Errors are the standard errors for the regression analyses.

Supplementary Table III: Tissue mol% FC and CE Content in WT vs. Scarb1$^{-/-}$ and Male vs. Female mice.[a]

	Mol% Tissue FC				CE Content, µg/mg protein			
	WT		Scarb1$^{-/-}$		WT		Scarb1$^{-/-}$	
Tissue	Female	Male	Female	Male	Female	Male	Female	Male
Plasma*	34.1±2.0 (12)	27.9±3.4 (10)	**57.7±1.8**† (11)	**65.9±1.3**† (5)	1.23±0.05 (12)	**1.91±0.10**‡ (10)	**2.54±0.08**† (11)	**2.51±0.13**† (5)
HDL*	21.4±1.3 (12)	22.4±2.6 (10)	**59.4±3.2**† (11)	**61.0±1.6**† (5)	1.23±0.05 (12)	**1.79±0.10**‡ (10)	**2.18±0.12**†(11)	**2.26±0.14**†(5)
Erythrocytes	49.6±0.4 (6)	48.8±0.5 (4)	**61.2±0.7**† (6)	**59.7±0.5**† (8)	0.12±0.03 (6)	0.16±0.04 (4)	0.16±0.06 (6)	**0.42±0.05**†‡(8)
Heart	16.8±0.2 (12)	16.7±0.5 (10)	**20.4±0.3**† (11)	**20.2±0.6**† (5)	0.97±0.99 (12)	bd (10)	1.95±0.93 (11)	bd (5)
Lung	38.4±0.4 (12)	38.8±0.3 (10)	**44.3±0.8**† (11)	**45.1±0.7**† (5)	1.53±0.59 (12)	1.34±0.55 (10)	**9.39±1.51**† (11)	**9.32±1.00**† (5)
Liver	19.8±0.4 (12)	20.8±0.6 (10)	**22.1±0.5**† (11)	**19.6±0.2**‡ (5)	1.86±0.98 (12)	4.63±1.31 (10)	5.90±1.96 (11)	3.14±3.01 (5)
Abdominal Fat	27.7±3.3 (4)	22.0±2.4 (5)	28.1±1.4 (4)	**36.4±3.3**† (5)	3.3±4.7 (4)	**25.5±3.2**$^{¥}$ (5)	bd (4)	**48.4±9.6**$^{\dagger\ddagger¥}$ (5)
Ovarian Fat	24.5±1.2 (5)		22.5±1.2 (4)		bd (5)		**7.77±4.2**† (4)	
Testes Fat		13.4±1.4 (5)		**19.3±2.7**† (5)		4.19±1.1 (5)		**26.3±5.8**† (5)
Ovaries	38.6±3.7 (12)		**69.0±2.3**† (8)		**179.3±9.4 (12)**		**76.0±6.3**† (8)	
Testes		27.0±0.3 (10)		26.1±0.2 (5)		7.0±0.6 (10)		5.5±0.4 (5)
Adrenals	27.2±0.4 (3)	**11.7±5.9** (3)$^{¥}$	28.9±6.0 (3)	**18.5±1.2** (7)‡	**305.1±5.5** (3)	**244.3±19.8** (3)‡	**48.5±16.1** (3)†	**13.5±1.0** (7)†‡
Brain	50.6±0.7 (12)	49.8±0.5 (10)	50.5±0.4 (11)	50.0±1.3 (5)	19.1±1.4 (12)	18.7±1.1 (10)	19.0±1.3 (11)	20.5±1.3 (5)
Kidney	30.8±0.4‡ (12)	**24.4±0.4**‡ (10)	31.1±0.4‡ (11)	**25.2±0.6**‡ (5)	0.73±0.30 (12)	1.09±0.19 (10)	0.64±0.33 (11)	0.64±0.19 (5)
Spleen	45.7±0.7 (12)	44.8±0.7 (10)	45.7±0.8 (11)	46.6±0.5 (5)	bd	bd	bd	2.68±0.7†‡ (5)

[a]Values are mean ± (SEM). Number of mice per group is given in parentheses (n). Red font, Scarb1$^{-/-}$ values differ from WT; blue font, male vs. female values differ; bd, below detection limit.

*Plasma and HDL CE values are mg CE/mL plasma.

†Values significantly different between genotypes for the same sex, $p<0.05$.

‡Values significantly different between sexes for the same genotype, $p<0.05$.

$^{¥}p<0.10$ for Scarb1$^{-/-}$ M vs WT-M, and for WT-M vs -F for abdominal fat CE, and for WT-M vs -F adrenal mol % FC.

Supplementary Table IV: Kinetic Constants for HDL-[^{3}H]FC Clearance In Vivo*

Genotype/Sex	k_i, %/min	k_1, min^{-1}	$t_{1/2}$, min	Final % DPM	ΔHDL-FC, mg/mL/min	FCR, pools/min*
WT Female	42.8 ± 1.6	1.27 ± 0.28	0.6	7.5 ± 1.8	0.055	1.16
WT Male	35.3 ± 2.0†	0.72 ± 0.07	1.1	8.9 ± 1.5	0.070	0.63
Scarb1$^{-/-}$ Female	26.9 ± 1.7‡	0.86 ±0.59	1.9	37 ± 6	0.28	0.36
Scarb1$^{-/-}$ Male	20.8 ± 1.8†‡	0.59 ± 0.38	3.0	40 ± 7	0.28	0.23

*Rate constants were from a fit of the data in **Figure 6**, where k_i is the initial rate constant, k_1 is the rate constant from the exponential fit of the data over 0 – 90 minutes, $t_{1/2} = \ln 2/k_1$, and Final %DPM is the asymptote of the exponential fit (**Figure 6**). Initial ΔHDL-FC is the initial %change/min (**Figure 6**) times the plasma HDL-C concentration (**Figure 1**). FCR (fractional catabolic rate) = $0.693/t_{1/2}$. Standard errors are from the regression analyses.
†Male vs Female, $p<0.02$. ‡†
‡Scarb1$^{-/-}$ vs WT, $p<0.001$.

Supplementary Table V: Differences in Tissues Compositions and Plasma Kinetics according to Sex*

Analyte	Comparison by Sex
Plasma-PL	WT M > WT F
Plasma-CE	WT M > WT F
HDL-CE	WT M > WT F
Erythrocyte-PL	Scarb1$^{-/-}$ M > Scarb1 F
Erythrocyte-TC	Scarb1$^{-/-}$ M > Scarb1 F
Erythrocyte-FC	Scarb1$^{-/-}$ M > Scarb1 F
Erythrocyte-CE	Scarb1$^{-/-}$ M > Scarb1 F
Erythrocyte-FC/TC	Scarb1$^{-/-}$ M < Scarb1 F
Heart-TC	Scarb1$^{-/-}$ M < Scarb1 F
Heart-CE	Scarb1$^{-/-}$ M < Scarb1 F
Heart-FC/TC	Scarb1$^{-/-}$ M > Scarb1 F
Lung-PL	WT M < WT F
Lung-FC	WT M < WT F
Liver-PL	Scarb1$^{-/-}$ M < Scarb1 F
Liver-TC	Scarb1$^{-/-}$ M < Scarb1 F
Liver-FC	Scarb1$^{-/-}$ M < Scarb1 F
Liver-TG	Scarb1$^{-/-}$ M < Scarb1 F
Liver-mol% FC	Scarb1$^{-/-}$ M < Scarb1 F
Abdominal Fat-PL	Scarb1$^{-/-}$ M > Scarb1 F
Abdominal Fat-TC	Scarb1$^{-/-}$ M > Scarb1 F
Abdominal Fat-CE	Scarb1$^{-/-}$ M > Scarb1 F
Abdominal Fat-FC/TC	Scarb1$^{-/-}$ M < Scarb1 F
Adrenal-PL	Scarb1-/- M < Scarb1 F; WT M < WT F
Adrenal-TC	Scarb1-/- M < Scarb1 F; WT M < WT F
Adrenal-FC	Scarb1-/- M < Scarb1 F; WT M < WT F
Adrenal-CE	Scarb1-/- M < Scarb1 F; WT M < WT F
Adrenal-mol% FC	Scarb1-/- M < Scarb1 F; WT M < WT F
Adrenal FC/TC	WT M < WT F
Kidney-PL	WT M > WT F
Kidney-TC	Scarb1-/- M < Scarb1 F
Kidney-FC	Scarb1-/- M < Scarb1 F
Kidney-mol% FC	Scarb1-/- M < Scarb1 F; WT M < WT F
Spleen-CE	Scarb1-/- M > Scarb1 F
Spleen-FC/TC	Scarb1-/- M < Scarb1 F
Plasma FC clearance rate	Scarb1-/- M < Scarb1 F; WT M < WT F

*Red and black font distinguish F vs. M significant differences among Scarb1$^{-/-}$ vs. WT mice respectively.

Supplementary Table VI: Major Resources

Animals (in vivo studies)

Species	Vendor or Source	Background Strain	Sex	Persistent ID / URL
Mus musculus	The Jackson Laboratory	B6;129S-Scarb1tm1Kri/J and WT C57BL/6J	M & F	JAXMice Search

Cultured Cells

Name	Vendor or Source	Sex (F, M, or unknown)	Persistent ID / URL
J774 macrophages	American Tissue Culture Collection	NA	ATCC: The Global Bioresource Center

Materials and Methods

Disclosure: The data that support the findings of this study are available from the corresponding author upon reasonable request.

Lipoprotein Isolation: Lipoproteins were isolated from pooled mouse plasma (5-10/genotype) by sequential flotation.[1, 2] Purity was verified by size exclusion chromatography (SEC) [3] and compositional analyses. HDL from individual mice was isolated by heparin-manganese precipitation of plasma APOB lipoproteins.[4, 5] Plasma and tissue lipids were determined using enzyme-based assays for FC, total cholesterol (TC), PL, and triglyceride (TG) (Fujifilm Wako Diagnostics Inc.). Cholesterol ester (CE) concentrations were calculated as (mg TC - mg FC) x 1.6. . Protein was determined by the DC Protein Assay (Bio-Rad, Inc.).

In Vitro HDL-FC Transfer Kinetics: HDL was radiolabeled with [^{3}H]FC as described previously.[6] In brief, [^{3}H]FC in ethanol was transferred to filter paper (~1 cm^2) and the solvent evaporated. The labeled filter paper was transferred to incubation buffer (TBS; 10 mM Tris, 100 mM NaCl, containing 1 mg protein/mL of WT, Scarb1$^{-/-}$ or human HDL and incubated at 4˚C overnight. The filter paper was then removed. Specific activities (dpm/nmoles FC) were calculated on the basis of HDL-FC concentration and β-counting of aliquots in triplicate. According to SEC, HDL-absorbance (280 nm) and radiolabel co-eluted. To assay rates of transfer of HDL-FC to LDL, HDL-[^{3}H]FC (10 µg protein/mL) was incubated with human LDL (1.0 mg/mL) at 37 °C. Aliquots were removed over time (0 – 20 min) and LDL precipitated with heparin-Mn^{+2};[4, 5]. Supernatants containing HDL were β-counted and counts vs. time data from two independent experiments were combined and fitted to a three-parameter exponential decay equation from which transfer rate constants and the asymptote, i.e., the equilibrium [^{3}H]FC transferred to LDL, were extracted.

Equilibrium Distribution of [^{3}H]FC between LDL and HDL: Given that HDL-[^{3}H]FC spontaneously transfers to LDL with $t_{1/2}$ ~5 min,[4] we measured the concentration-dependence of this process at equilibrium by incubating human LDL (0.8 mg protein/mL) and various concentrations of HDL-[^{3}H]FC (0.4 to 2.0 mg protein/mL) for ~18 h at 37 °C. At the end of the incubations, aliquots were passed over a size exclusion column, which separates HDL from LDL. The eluted fractions were collected and β-counted, and the mass of [^{3}H]FC transferred from HDL to LDL was based on the radioactivity eluting with LDL. Genotype-dependent differences in the HDL-[^{3}H]FC to LDL transfer were determined by comparing the slopes of a linear regression analysis of LDL-associated FC mass vs. the HDL-protein and HDL-FC concentrations in the incubation.

FC Flux between HDL and Macrophages: FC flux between HDL and J774 macrophages (J774A.1, ATCC® TIB67™) were measured essentially as described.[3, 7-10] To quantify FC influx, [^{3}H]FC-labeled HDL was incubated with macrophages and cell-associated [^{3}H]FC was measured as a function of HDL concentration and time.[3, 10] Cells were seeded in 12-well plates and each assay point was performed in triplicate. At various incubation times, media were collected, and the cells washed three times with cold buffer. Cell lipids were twice extracted with isopropanol and the extracts β-counted. Residual cells were solubilized in 1 mL NaOH (0.1 M) and the protein quantified (BioRad DC). Influx dose-response was assayed at 2 h and analyzed using a two-parameter hyperbolic function, Influx (FC/mg cell-protein) = ac/(b + c), where c is HDL concentration, a is maximum uptake, and b, the HDL concentration that produces 50% of maximum uptake, is a measure of influx power. For time course experiments, [^{3}H]FC-labeled HDL (20 µg/mL) was incubated with macrophages for various times. Lipid

extraction and protein assays were performed as above. Uptake kinetics were fitted to a rising exponential function, Influx (FC/mg cell-protein) = $a(1-e^{-kt})$, where t is time, k is the rate constant, and a is maximum uptake.

FC efflux to HDL was measured by incubating HDL with [^{3}H]FC-labeled macrophages as described.[7] J774 macrophages were seeded at 0.2 x 10^6 cells/3.8cm^2, grown to 40% confluency and radiolabeled with [^{3}H]FC (0.5 µCi/well). The media were removed, and the cells washed. For dose-response, HDL (10-100 µg HDL protein/mL) was added to the media, cells were incubated for 2 h, and for time course experiments the HDL concentration was 20 µg protein/mL, after which media and washed cells were collected. The media were centrifuged (5 min @ 13,200 rpm) to remove cell debris and β-counted. Cell lipids were β-counted, and cell protein determined as above. Respective time- and concentration-dependence of efflux were fitted to exponential and hyperbolic equations as described for influx. Initial rates of influx and efflux were determined from the fitted time-course equations.

Mouse Management and Tissue Analysis: Scarb1$^{-/-}$- and WT C57BL/6J-mice (The Jackson Laboratory) were maintained on normal laboratory diet (Teklad Envigo Cat 2920). All were studied at 12-25 weeks of age except those analyzed for adrenal lipid compositions, which were 8-40 weeks old. Numbers of mice used for the various analyses are given in the Figure and Table legends. All mouse procedures complied with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee. Retro-orbital injections of HDL-[^{3}H]FC were conducted immediately after a one-time isoflurane inhalation (2.5%, 3-5 minutes) to achieve an anesthetic effect. WT and Scarb1$^{-/-}$ mice were euthanized and their blood collected by heart puncture into EDTA; tissues were harvested for lipid and protein analyses.[11-13] Tissues were weighed, homogenized, and extracted (hexane:2-propanol:acetic acid:3:2:1% v/v/v). Tissue-protein was solubilized with 0.4M NaOH + 1% sodium dodecyl sulfate. Extracted lipids were dissolved in 1% Triton in chloroform, the chloroform evaporated under nitrogen, and the lipids solubilized in water. Compositions were expressed as lipid mass/protein mass.

In Vivo HDL-FC Metabolism: HDL-FC turnover kinetics were determined in WT and Scarb1$^{-/-}$ mice (The Jackson Laboratory) as described.[6] Mice (n = 3-4/time point) were retro-orbitally injected with HDL-[^{3}H]FC, and euthanized at various times post-injection. Blood was collected and centrifuged to sediment cells, and the plasma from the supernatant was β-counted. Erythrocytes were washed, lipid-extracted, and protein determined as above. Kinetic data were fitted to a two-parameter exponential function of percent injected dose vs. time; initial rates were calculated as the percent change/min between t = 0 and 2 min.

Statistical Analysis: Data, presented as mean ± standard deviation (SD) or standard error of the mean (SEM) are given in Table and Figure legends. Group means were compared by Student's t-test in Prism 8.0 or Microsoft Excel (Office 16). Linear and non-linear regression analyses were done using SigmaPlot 12.0 and Prism 8.0. Prism was used to compare linear regression slopes and intercepts, including log transformed data for exponential decay fits. Differences in the plasma, lipoprotein, and tissue lipid compositions of WT male and female vs. Scarb1$^{-/-}$ male and female mice were identified by pairwise comparisons when the analysis of variance (SigmaPlot 12.0) on all four groups indicated significant differences, $p<0.05$. Data were tested for normality (Shapiro-Wilk) and Equal Variance, and if passed, pairwise comparisons between groups were done using the All Pairwise Multiple Comparison Procedures (Holm-Sidak method) or Student's t-test. If the data failed normality or equal variance tests, the Kruskal-Wallis one-

way analysis of variance on ranks or a rank-sum test were used for pairwise comparisons and calculation of p values. Because in some tissues, lipid values for male and female mice of the same genotype differed, for pairwise comparisons of genotypes, we report only comparisons for the same sex, i.e. WT-F vs Scarb1$^{-/-}$-F and WT-M vs Scarb1$^{-/-}$M. $P < 0.05$, which was considered significant, are given in the tables and figure legends. Data for human HDL when provided is for comparison and was not statistically evaluated.

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